

Prognostic Significance of Cyclooxygenase 2 mRNA Expression in Non-Small Cell Lung Cancer

Jan Brabender, MD,*‡ JiMin Park, MS,* Ralf Metzger, MD,‡ Paul M. Schneider, MD,‡ Reginald V. Lord, MD,† Arnulf H. Hölscher, MD,‡ Kathleen D. Danenberg, MS, and * Peter V. Danenberg, PhD*

From the *Department of Molecular Biology and Biochemistry and USC/Norris Comprehensive Cancer Center, Los Angeles, California; the †Department of Surgery, University of Southern California Keck School of Medicine, Los Angeles, California; and the ‡Department of Visceral and Vascular Surgery, University of Cologne, Germany

Objective

To investigate cyclooxygenase-2 (COX-2) mRNA expression in curatively resected non-small cell lung cancer (NSCLC) and to determine its association with prognosis.

Summary Background Data

Lung cancer is one of the most common malignancies in the world. Despite improvements in the diagnosis and treatment of NSCLC, the 5-year survival rate remains less than 15%. Identification of prognostic predictors based on molecular alterations could lead to additional diagnostic tools and eventually to more effective therapeutic options. Overexpression of COX-2 has been reported in several human malignancies, including lung cancer, but the prognostic importance of this overexpression has not been elucidated.

Methods

COX-2 mRNA expression was analyzed using a quantitative real-time polymerase chain reaction (Taqman) method in sur-

gically resected tumor specimens from 89 patients with curatively resected NSCLC.

Results

COX-2 mRNA was detectable in all 89 (100%) tumor tissues. High COX-2 expression in tumors was significantly associated with inferior survival. Multivariate analysis showed that high COX-2 expression is an independent predictor of worse survival in patients with NSCLC.

Conclusions

High COX-2 mRNA expression is an important biomarker for biologically aggressive disease in NSCLC and might be helpful in identifying patients who would benefit from additional therapies for controlling their disease.

Lung cancer is the most common cause of cancer-related death in the United States, accounting for more deaths than from prostate, breast, and colorectal cancer combined.¹ Each year approximately 170,000 new cases of lung cancer are diagnosed in the United States.¹ Radical surgery offers the only chance for cure in patients with non-small cell lung cancer (NSCLC), but despite improvements in the detection and treatment of lung cancer in the past two decades, the

5-year survival rate remains less than 15%.² To improve the outcome of patients with NSCLC, the development of a prognostic classification based on molecular alterations will be crucial. Such a classification could provide additional accurate and useful diagnostic tools and, eventually, more effective therapeutic options.

Epidemiologic studies have shown that prolonged use of nonsteroidal antiinflammatory drugs (NSAIDs) reduces the risk of colon cancer,^{3–5} and other studies have suggested that aspirin may also reduce the incidence of lung cancer.⁶ The best-known target of NSAIDs, including aspirin, is the enzyme cyclooxygenase (COX), a key enzyme involved in the production of prostaglandins and other eicosanoids from arachidonic acid.^{7,8} Two COX isoforms, COX-1 and COX-2, have been identified. Whereas COX-1 is considered a constitutively expressed housekeeping gene, COX-2 is an in-

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Correspondence: Jan Brabender, MD, Department of Surgery, University of Cologne, Joseph-Stelzmann Str. 9, 50931 Cologne, Germany.

E-mail: drbrabender@cs.com

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ducible immediate-early gene associated with inflammation and carcinogenesis.^{8–10} Overexpression of *COX-2* has been reported in several human malignancies, including colorectal cancer,¹¹ gastric cancer,¹² breast cancer,¹³ esophageal carcinoma,¹⁴ and lung cancer.^{15–19} However, the prognostic role of *COX-2* protein expression in NSCLC remains controversial. Although Achiwa et al¹⁷ reported an association between *COX-2* overexpression and survival in a patients with stage 1 adenocarcinoma of the lung, Marrogi et al¹⁹ were unable to detect any association between *COX-2* expression and clinical outcome in patients with NSCLC. No studies concerning the prognostic role of *COX-2* mRNA expression in NSCLC have been reported.

To determine the prognostic relevance of *COX-2* mRNA expression in NSCLC, we performed quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR; Taqman)^{20,21} on surgically removed tumor specimens from 89 patients with curatively resected NSCLC.

PATIENTS AND METHODS

Patients and Specimens

Tumor specimens from 89 patients with NSCLC, available from a previous prospective clinical trial of 103 consecutive patients,²² were included in this study. There were 67 (75%) men and 22 (25%) women, with a median age of 64 years (range 34–83). Forty-one (46%) patients had squamous cell carcinomas, 33 (37%) had adenocarcinomas, and 15 (17%) had large cell carcinomas. The primary tumors were graded histopathologically as well-differentiated (G1, one patient), moderately differentiated (G2, 19 patients), and poorly differentiated (G3, 69 patients). Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification:²³ 44 patients (50%) had stage 1 tumors, 18 (20%) had stage 2 tumors, and 27 (30%) had stage 3a tumors. All 89 patients underwent thoracic surgery. All tumors were radically removed (R0 resection) by lobectomy (n = 57), bilobectomy (n = 11), pneumonectomy (n = 11), and extended pneumonectomy (n = 10) including mediastinal lymphadenectomy. Patients with histopathologic stage 3a tumors received postoperative radiotherapy. Informed consent was obtained from each patient.

The median follow-up was 85.9 months (range 63–105), and no patient was lost to follow-up. Tissue for gene expression analysis was obtained during surgery immediately after lung resection and before starting mediastinal lymphadenectomy. The tissues were immediately frozen in liquid nitrogen and stored at -80°C . Six-micrometer frozen sections were taken from blocks of tumor tissue, and starting with the first section, every fifth section was routinely stained with hematoxylin and eosin and evaluated histopathologically. Sections were pooled for analysis from areas estimated to have at least 75% malignant cells.

mRNA Isolation

Total RNA was isolated by a single-step guanidinium isothiocyanate method using the QuickPrepMicro mRNA Purification Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer's instructions. After RNA isolation, cDNA was prepared from each sample as described previously.²⁴

Real-Time Polymerase Chain Reaction Quantification

Quantitation of cDNA was done using a fluorescence based real-time detection method (ABI PRISM 7700 Sequence Detection System [Taqman], Applied Biosystems, Foster City, CA) as previously described.^{20,21} The PCR reaction mixture consisted of 600 nmol/L of each primer, 200 nmol/L probe, 2.5 U AmpliTaq Gold Polymerase, 200 $\mu\text{mol/L}$ each dATP, dCTP, dGTP, 400 $\mu\text{mol/L}$ dUTP, 5.5 mmol/L MgCl_2 , and $1 \times$ Taqman Buffer A containing a reference dye, to a final volume of 25 μL (all reagents Applied Biosystems). Cycling conditions were 50°C for 10 seconds and 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute.

The primers and probe sequences used were as follows: *COX-2* primers: GCTCAAACATGATGTTTGCATTC and GCTGGCCCTCGCTTATGA; probe 6FAM (carboxyfluorescein) 5'-TGCCCAGCACTTCACGCATCAGTT-3'TAMRA(N,N,N',N'-tetramethyl-6carboxyrhodamine); and β -actin primers: TGAGCGCGGCTACAGCTT and TCCTTAATGTCACGCACGATTT; probe: 6FAM5'-ACCACCACGCCGAGCG G-3'TAMRA.

Statistical Analysis

Taqman analyses yield values that are expressed as ratios between two absolute measurements (gene of interest/internal reference gene). The chi-square test was used to analyze the association between categorical clinicopathologic data and *COX-2* expression status. Hazard ratios were used to calculate the relative risks of death. These calculations were based on the Pike estimate, with the use of the observed and expected number of events as calculated in the log-rank test statistic.²⁵ The maximal chi-square method of Miller and Siegmund²⁶ and Halpern²⁷ was adapted to determine which expression value best segregated patients into poor- and good-prognosis subgroups (in terms of likelihood of surviving), with the log-rank test and the stratified log-rank test used to measure the strength of the grouping. To determine a *P* value that would be interpreted as a measure of the strength of the association based on the maximal chi-square analysis, 1,000 boot-straplike simulations were used to estimate the distribution of the maximal chi-square statistics under the hypothesis of no association.²⁷ Multivariate analysis was performed with the Cox proportional hazards re-

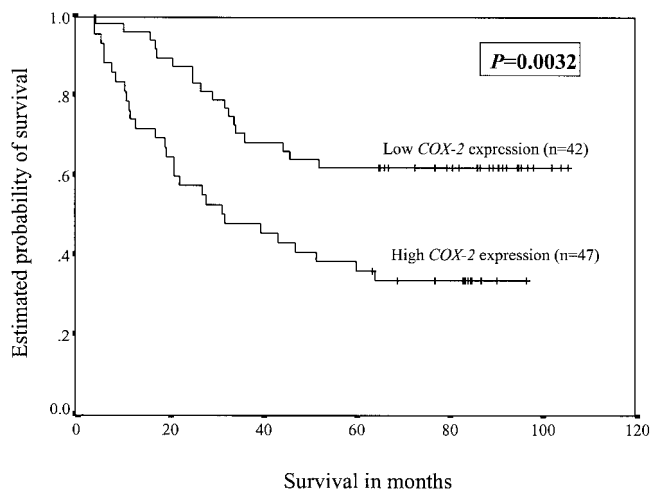


Figure 1. Survival of patients with non-small cell lung cancer as a function of the amount of *COX-2* mRNA expression (the ratio between *COX-2* and the internal reference gene β -actin) in specimens of primary non-small cell lung cancer. High *COX-2* mRNA levels constituted ratios greater than 0.6; low levels were ratios equal to or less than 0.6.

gression model. The level of significance was set to $P < .05$. All P values reported were based on two-sided tests.

RESULTS

COX-2 mRNA expression was detectable by quantitative real-time PCR (Taqman) in all 89 (100%) tumor specimens. The median *COX-2* mRNA expression, expressed as a ratio to the internal reference gene β -actin, was 0.85 (range 0.02–15.77).

With a median follow-up of 85.9 months for the 89 patients analyzed in this study, the median survival was 59.7 months (range 3.8–105.3). The maximal chi-square statistic of Miller and Siegmund²⁶ and Halpern²⁷ was adapted to determine which *COX-2* mRNA expression value best segregated patients into poor- and good-prognosis subgroups. This method found that segregation for *COX-2* mRNA levels was best achieved with a relative expression level of 0.6. By this criterion, 47 (53%) patients had a low *COX-2* expression status and 42 (47%) patients had a high *COX-2* expression status. Table 1 shows associations between clinicopathologic data and *COX-2* expression status. No statistically significant associations were detectable.

Median and 5-year survival rates depending on various clinical variables and *COX-2* expression status are summarized in Table 2. The median survival for patients with a high *COX-2* mRNA expression status was 31.1 months (95% confidence interval 8.72–53.48), whereas the median survival for those with a low *COX-2* mRNA expression status was not reached ($P = .0032$, log-rank test). The respective survival curves are presented in Figure 1 and show 5-year survival rates of $31.2\% \pm 7.7$ for patients with high *COX-2* expression levels and $61.7\% \pm 7.1$ for those with low *COX-2* expression levels ($P = .0025$). Further, by

stratifying patients by stage (stage 1 vs. stage 2 vs. stage 3a), *COX-2* mRNA expression was shown to be an even stronger predictor of survival for curatively resected NSCLC ($P = .0002$, stratified log-rank test).

The importance of *COX-2* as a prognostic factor was next determined by the Cox proportional hazards model analysis. Two logistic regression models were tested. Model A included the parameters age, gender, histopathologic type, UICC TNM tumor stage, and *COX-2* expression status. Model B included the pT and pN categories instead of histopathologic tumor stage. Significant independent prognostic factors were shown to be UICC TNM tumor stage ($P < .001$) and *COX-2* ($P < .001$) expression status in model A, and the pN categories ($P < .001$) and *COX-2* expression status ($P = .013$) in model B. Table 3 shows the statistically significant parameters in the two regression models.

DISCUSSION

To test whether the level of *COX-2* expression is of prognostic importance in NSCLC, we used a quantitative real-time RT-PCR (Taqman) method to analyze *COX-2* mRNA expression in tumor specimens of 89 patients with curatively resected NSCLC. Expression of *COX-2* was detectable in all (100%) specimens analyzed; however, the intratumoral content of *COX-2* mRNA expression varied over a 788-fold range. This observation of seemingly variable amounts of mRNA implies heterogeneity of expression patterns within individual tumor cells and may reflect the biologic behavior of these tumors.

Overexpression of *COX-2* has been reported in human NSCLC,^{16,18,19} and previous studies have shown that *COX-2* overexpression may alter the biologic behavior of tumor cells in a number of ways.^{28–30} Tsujii et al²⁹ have shown that constitutive expression of *COX-2* could result in phenotypic changes that enhance the metastatic potential of colorectal cancer cells, leading to an increased invasiveness. In analogy to colorectal carcinomas, Hida et al¹⁵ reported that a marked increase in *COX-2* immunoreactivity in NSCLC, specifically adenocarcinomas, was associated with tumor-invasive lesions and lymph node metastasis, suggesting that increased *COX-2* expression may be associated with an invasive and more aggressive phenotype in this disease. Indeed, Achiwa et al¹⁷ were the first to show a prognostic significance of elevated *COX-2* expression in human cancer. Although no association between elevated *COX-2* protein expression and clinical outcome in stage 1 to 3 adenocarcinomas of the lung was detectable, high *COX-2* expression correlated with inferior survival in a subgroup of adenocarcinomas, namely stage 1 disease. In contrast to our findings, Marrogi et al¹⁹ recently reported no association between *COX-2* protein expression and clinical outcome in patients with NSCLC. There are several possible explanations for these discordant findings. First, Marrogi et al investigated *COX-2* protein expression, whereas we examined *COX-2* expression at the mRNA level. Second, immunohistochem-

ical methods are semiquantitative compared with the quantitative RT-PCR technique used in our study. Third, the study population analyzed by Marrogi et al¹⁹ consisted of patients with stage 1 to 4 NSCLC, whereas only patients with curatively resected NSCLC stage 1 to 3a were included in our study.

The most striking finding in our study is the association between high *COX-2* mRNA expression levels and worse survival in patients with curatively resected NSCLC. This finding may be clinically important, particularly because our study population represents a large number of patients with different histopathologic subtypes of lung cancer, in all of whom curative surgery was achieved. Our observation adds another step toward the development of a molecular classification of NSCLC and suggests that quantitation of *COX-2* mRNA expression might help identify NSCLC patients at higher risk of early disease recurrence who will benefit from additional therapy to control their disease.

Previous studies have shown that treatment with NSAIDs decreases cell growth and induces apoptosis in NSCLC in vitro.^{31,32} Of particular interest is that the responsiveness to selective *COX-2* inhibitors was influenced by the degree of *COX-2* expression.³¹ The availability of selective *COX-2* inhibitors gives our results additional importance because increased *COX-2* expression might represent a direct therapeutic target in patients with NSCLC. Further studies investigating selective *COX-2* inhibition in patients with NSCLC are warranted to gain more insight into the potential clinical usefulness of this therapeutic approach.

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